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Coordinated effects of distal mutations on environmentally coupled tunneling in dihydrofolate reductase

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Contributed by Stephen J. Benkovic, August 23, 2006

One of the most intriguing questions in modern enzymology is whether enzyme dynamics evolved to enhance the catalyzed chemical transformation. In this study, dihydrofolate reductase, a small monomeric protein that catalyzes a single C-H-C transfer, is used as a model system to address this question. Experimental and computational studies have proposed a dynamic network that includes two residues remote from the active site (G121 and M42). The current study compares the nature of the H-transfer step of the WT enzyme, two single mutants, and their double mutant. The contribution of quantum mechanical tunneling and enzyme dynamics to the H-transfer step was examined by determining intrinsic kinetic isotope effects, their temperature dependence, and activation parameters. Different patterns of environmentally coupled tunneling were found for these four enzymes. The findings indicate that the naturally evolved WT dihydrofolate reductase requires no donor-acceptor distance fluctuations (no gating). Both single mutations affect the rearrangement of the system before tunneling, so some gating is required, but the overall nature of the environmentally coupled tunneling appears similar to that of the WT enzyme. The double mutation, on the other hand, seems to cause a major change in the nature of H transfer, leading to poor reorganization and substantial gating. These findings support the suggestion that these distal residues synergistically affect the H transfer at the active site of the enzyme. This observation is in accordance with the notion that these remote residues are part of a dynamic network that is coupled to the catalyzed chemistry.

enzyme dynamics | hydrogen transfer | hydrogen tunneling | kinetic isotope effect | structure function

The mechanisms by which enzymes catalyze chemical reactions have been studied extensively. Yet, the question of whether enzyme dynamics evolved to enhance enzymatically catalyzed chemical reactions remains open. To avoid terminology confusion, the term dynamics, as used in this work, needs to be defined. Several researchers construe dynamics only as nonequilibrium motions along the reaction coordinate (1), whereas most enzymologists interpret dynamics as any motion in the reaction's environment (2, 3). In this article, we use the latter definition and address the possibility that dynamics of the whole protein (not only of its active site) play a role in catalysis. Dihydrofolate reductase (DHFR) was chosen as a model system because it is a small enzyme and its dynamics have been studied experimentally and theoretically. DHFR catalyzes a simple chemical transformation (C-H-C transfer) that can be examined experimentally in great detail. Hence, changes in protein dynamics and their effects on the chemical step can be examined and related to the catalytic activation of the C-H bond.

DHFR is a flexible, monomeric protein. Fig. 1 presents the structure of *Escherichia coli* DHFR (ecDHFR) and highlights the active site and the two residues under investigation (G121 and M42). The enzyme catalyzes the reduction of 7,8-dihydrofolate (H₂F) to 5,6,7,8-tetrahydrofolate (H₄F) with the stereospecific transfer of a hydride from the *pro-R* C4 position of the nicotinamide ring to the *si* face of the C6 of the pterin ring (2). DHFR has served

as a platform for many theoretical and experimental studies, a few of which are discussed below.

The kinetic mechanism of ecDHFR was derived from equilibrium-binding, steady-state, and presteady-state kinetic studies (2, 4). These studies revealed a rather complex kinetic cascade within which the step that includes hydride transfer is mostly rate-limiting at high pH. Similar kinetic schemes have been drawn for various mutants of ecDHFR (5–14), setting the framework for studies of the relationship between kinetics and dynamics.

Changes in protein dynamics in response to ligand binding, substrate turnover, and mutagenesis have been probed by using numerous experimental and theoretical approaches. Structures of ecDHFR obtained by x-ray diffraction studies in unbound form and in binary and ternary complexes with various ligands suggest that the enzyme assumes open, closed, and occluded conformations along the reaction pathway (15). NMR relaxation experiments confirmed the conformational changes involving these loops and indicated that binding of the substrate and cofactor induces such changes both in and distal to the active site during the catalytic cycle (16–18). These studies probed the distribution of conformational ensembles that ecDHFR assumes and addressed the role of the different ensembles in catalysis. NMR studies with G121V (one of the mutants studied here) suggested that G121 affects catalysis by altering the distribution of conformational ensembles (14, 16–19).

Theoretical studies have further enhanced our understanding of the role of enzyme structure and dynamics and the impact of mutations on enzyme catalysis. Classical molecular dynamics simulations (20–22) have been used to identify correlated and anticorrelated motions within many of the same regions implicated by the NMR relaxation experiments (16–18). These correlations exist in the reactant complex, but are diminished in the product complex, which implies a possible role of dynamics in catalysis. Distal ecDHFR mutants with reduced activities exhibit reduced correlated motions compared with the WT enzyme. More recently, hybrid quantum/classical molecular dynamics simulations have implicated a network of coupled motions extending throughout the entire protein and its ligands (23–28). These coupled motions, representative of equilibrium, thermally averaged conformational changes along the reaction coordinate, lead to active site configurations that enhance the hydride transfer. These hybrid simulations have been expanded to constrained systems in which a constraint was placed on the distances between α -carbons of distal residues (29). The results suggest that freezing the motion between two distal residues can deteriorate the network of coupled motions and

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The authors declare no conflict of interest.

Abbreviations: DHFR, dihydrofolate reductase; ecDHFR, *Escherichia coli* DHFR; KIE, kinetic isotope effect.

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Table 2. Comparative activation parameters of initial velocity measurements at pH 9

Parameter	DHFR, kcal/mol			
	WT	G121V	M42W	G121V-M42W
E_a	5.56 ± 0.58	5.30 ± 0.12	5.09 ± 0.83	3.91 ± 0.18
ΔH^\ddagger	4.95 ± 0.58	4.69 ± 0.12	4.48 ± 0.83	3.30 ± 0.18
$T \cdot \Delta S^\ddagger$ (25°C)	-11.79 ± 0.56	-12.20 ± 0.41	-12.23 ± 0.80	-15.72 ± 0.38
ΔG^\ddagger (25°C)	16.74 ± 0.81	16.89 ± 0.43	16.71 ± 1.15	19.02 ± 0.42

where $^h(k_{\text{cat}}/K_M)_{\text{l-obs}}$ is the observed $1/h$ KIE on k_{cat}/K_M , and k_1/k_h is the intrinsic $1/h$ KIE on the H-transfer step. C_f represents the forward commitment to catalysis, which is the sum of the ratios between the rate of the forward, isotopically sensitive, hydride transfer step and each of the rates of the preceding, backward, isotopically insensitive steps.

Fig. 4 presents the forward commitment (C_f) values of the WT, G121V, M42W, and G121V-M42W DHFRs as Arrhenius plots (logarithmic scale of C_f vs. the reciprocal of the absolute temperature). The temperature dependencies of the observed KIEs and forward commitments (C_f) of the compared isozymes are quite diverse. The comparison of Figs. 3–5 illustrates that the intrinsic KIEs and their corresponding observed KIEs are not related to each other by any simple function. Apparently, the factors affecting the isotopically sensitive step and the other kinetic steps are affected differently by the mutation (as are all microscopic rate constants). This observation emphasizes that great caution is needed when analyzing measured KIEs and their temperature dependence and further underlines the importance of exposing intrinsic effects.

Marcus-Like Models and Environmentally Coupled Tunneling. The interpretation of the data from the methods used here depends on the availability of a theoretical model that can address rates, KIEs, and their temperature dependency. In many cases, models based on transition-state theory, assuming a 1D rigid potential surface, successfully reproduced temperature-dependent KIEs either with or without a tunneling correction (46). Those models can rationalize temperature-independent large KIEs providing there is no activation energy for the isotopically sensitive step (34, 47, 61). However, such models cannot explain temperature-independent small KIEs with a significant activation energy. In an attempt to explain experimental results with such KIEs, several phenomenological models were proposed in recent years that fall under the title Marcus-like models (e.g., refs. 34, 36, 51, 53, 54, and 62–64). These models were constructed based on a single kinetic step (the chemical step), and thus pertain to the experimental measurements described here. Although these different models originate from

different basic principles, they all share several mathematical and physical features. In short, these models suggest that (i) the hydrogen should be treated quantum mechanically throughout the reaction coordinate (including tunneling); (ii) fluctuations of the reaction's potential surface occur on a time scale similar to or slower than the hydrogen-transfer rate, and thus determine the overall rate of hydrogen transfer (the solvent coordinate is the reaction coordinate as described in refs. 50, 65, and 66); and (iii) these fluctuations can be treated as two orthogonal vibrations, one that represents fluctuations in the donor-acceptor distance (the q coordinate) and the second that represents changes in the system's symmetry (the p coordinate) as illustrated in Fig. 5. For more extensive discussion of such Marcus-like models see refs. 34 and 50.

A general description of such model can be demonstrated in the following rate equation:

$$k = C \cdot MT \cdot HTT, \quad [2]$$

where C is a constant with insignificant temperature dependence, MT is a Marcus term that is mostly isotopically insensitive with the general form of:

$$MT = e^{-(\Delta G^\circ + \lambda)^2/4\lambda RT}, \quad [3]$$

where λ is the reorganization (or preorganization) energy and ΔG° is the reaction's thermophilicity (the driving force for the reaction) (67, 68), R is the gas constant, and T is the absolute temperature. HTT is the tunneling term, which is sensitive to the mass of the isotopic atom and the distance between donor and acceptor energy wells in any conformation that enables significant tunneling. This term represents the H-tunneling probability and includes the Franck-Condon nuclear overlap integral between the donor and

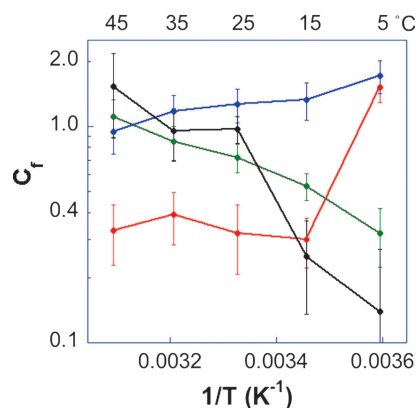


Fig. 4. Comparison of the Arrhenius plots of the commitment to catalysis (C_f) on k_{cat}/K_M for the WT (red; ref. 37), G121V (green; ref. 40), M42W (blue; ref. 41), and G121V-M42W (black) ecDHFRs.

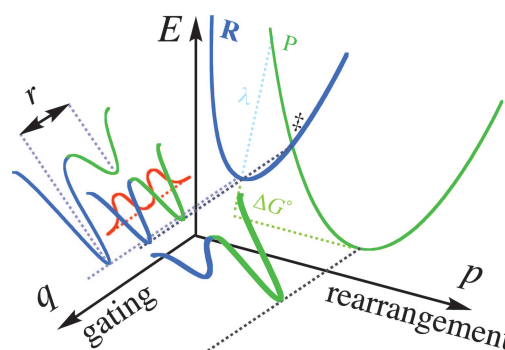


Fig. 5. Illustration of Marcus-like models showing energy surface of environmentally coupled hydrogen tunneling. Two orthogonal coordinates are presented: p , the environmental energy parabolas for the reactant state (R) and the product state (P) along which the rearrangement process take place; and q , the H-transfer potential surface at each p configuration. The donor-acceptor distance (r in Eq. 5) fluctuates along the q coordinate. In Marcus-like models this distance fluctuates harmonically around r_0 , thereby changing the tunneling probability [a phenomenon denoted gating by Klinman and co-workers (34, 69)]. In cases where r_0 is ideal for tunneling, the KIEs will be temperature independent. Otherwise, these thermal fluctuations will lead to the temperature dependency of the KIEs.

acceptor wave functions. It depends on the tunneling mass and the vibration state of the tunneling conformation (34, 52). *HTT* is an exponential function consisting of two terms. The first term is the integrated tunneling probability of all of the relevant donor–acceptor distances as a function of the isotopic mass (m_i) and frequency (ω_i). The second term is an exponential function of the energy involved in reaching each donor–acceptor distance (E_X) and is often defined as “gating.” An example of *HTT* would be (52):

$$HTT_i = \int_{r_1}^{r_0} e^{-m_i \omega_i \Delta r_i^2 / 2\hbar} e^{-E_X / RT} dX, \quad [4]$$

where Δr_i is the distance between the donor and acceptor wells at each conformation, ranging from the equilibrium distance (r_0) to r_1 , and X is the gating coordinate. The *HTT* is obviously isotopically sensitive and has an Arrhenius temperature dependency.

Mathematically speaking, all of these models separate the temperature dependence of the reaction rate (affected by both *MT* and *HTT*) from that of the KIEs (affected mostly by *HTT*). This feature enables Marcus-like models to accommodate systems with both temperature-dependent and temperature-independent KIEs, whether the activation barrier for the reaction is significant or not.

Various terms have been coined in these models to characterize hydride transfer in an enzymatic system, including “vibrationally enhanced tunneling” (53), “rate-promoting vibrations” (54), and “environmentally coupled tunneling” (69). In this article we use the terminology that was coined by Nagel and Klinman (34) and Franciso *et al.* (69), although other terms used by others, or newer terms by Klinman (70), are just as valid. Using this terminology, the thermally activated fluctuations of the system that alter the symmetry of the potential surface are referred to as “rearrangements” (the Marcus term, see Eq. 4) and the fluctuations of the donor–acceptor distance that actively modulate the tunneling barrier as gating (the Frank-Condon term, e.g., Eq. 5).

Rationalization of Current Findings. The temperature independence of the KIEs, with large A_i/A_h values, and nonzero E_a values for the WT *ecDHFR* (Fig. 3 and Table 1) has been rationalized in the context of a full tunneling Marcus-like model with ideal rearrangement of the potential surface (along the p coordinate in Fig. 5) (35). The average donor–acceptor distance in this system appears to be ideal for tunneling. That is to say, the environmental reorganization that must occur before tunneling can proceed has evolved to optimize reactive conformation for ground-state tunneling. Consequently, no thermally activated fluctuations along the q coordinate contribute to the tunneling rate and the KIEs are temperature independent (35). In this case, the observed E_a arises from the Marcus term (see Eqs. 2 and 3). For more details, see refs. 34 and 50, which discuss the Marcus-like models in great detail and describe how the temperature dependency of KIEs can be used as a probe for determining the coupling between the enzyme environment and the catalyzed reaction coordinate.

For both single mutants, the slightly inflated KIEs and their weak temperature dependence indicate that the rearrangement is not as perfect as for their WT counterpart, and that the average donor–acceptor distances are longer than that for the WT enzyme. Consequently, some thermally activated fluctuations along the q coordinate are required, leading to the slight temperature dependences of these KIEs (40, 41). Compared with that for G121V and M42W mutants, the steep temperature dependence of the KIEs for G121V–M42W *DHFR* suggests poor rearrangement and an average donor–acceptor distance that is too long to enable tunneling. Essential thermally activated gating fluctuations in this double mutant lead to the large temperature dependence of the KIEs.

The above comparison suggests that the average distance between the donor and the acceptor for hydride transfer is perfect for the WT, less perfect for the G121V and M42W mutants, and

significantly altered for the G121V–M42W mutant. Consequently, the hydride transfer with the G121V and M42W mutants seems to occur in a conformation closer to that of WT *DHFR* relative to the G121V–M42W mutant. These findings indicate that distal residues G121 and M42, both >15 Å away from the active site and 21 Å from each other, affect the hydride transfer at the active site in a synergistic fashion and constitute experimental support for the theoretical simulations suggesting that these residues are part of a dynamic network coupled to the catalyzed chemistry (24–26, 71).

Finally, a sequence-based statistical analysis (72) also indicated that these residues evolved in a coupled manner (28). Because functional coupling, as examined in the present work, may lead to an evolutionary bias, the relationships identified by the present study offer insight into the genetic coupling between G121 and M42.

Conclusions

The current work examines the effects of remote *DHFR* mutations (G121V and M42W) on the enzyme-catalyzed hydride transfer step by measuring KIEs and their temperature dependence. The results are interpreted in the framework of Marcus-like models. The findings for the G121V–M42W *ecDHFR* are compared with those for the G121V, M42W, and WT enzymes. The comparison indicates that the nature of the hydride transfer is slightly changed for the G121V and M42W mutants, but is significantly altered for the G121V–M42W double mutant. The WT *DHFR* reaction involves environmentally coupled H tunneling, which does not require thermally activated fluctuations of the donor–acceptor distance. Both single mutations mostly affect the prearrangement of the system before tunneling, leading to the observed slower reaction. The tunneling conformations of G121V and M42W were slightly altered to less ideal average tunneling conformations (r_0) relative to the WT, so some thermally activated gating fluctuations were required. The double mutant, on the other hand, demonstrated substantial changes in the nature of H transfer in accordance with the idea that these remote residues are part of a dynamic network that extends throughout the enzyme and are coupled to the catalyzed chemistry (24–26). The observation that most of the reduction in the double mutant’s H-transfer rate is entropic (Table 2) and the fact that only the double mutant has substantially temperature-dependent KIEs suggest that the simultaneous distortion of G121 and M42 affects the reorganization of the system (as expressed by ΔS^\ddagger) in a way that prohibits efficient ground-state tunneling. Because similar effects on each single mutant were much smaller (or negligible within experimental error), the current findings support a synergistic effect of G121 and M42 (both remote from the active site) on the catalyzed C–H transfer, in accordance with the proposed network of coupled motions that may have evolved to be coupled to the catalyzed reaction (25–28).

The importance of protein dynamics in enzymatic reactions has implications for protein engineering and rational drug design. Apparently, not only the catalytic active site should be mimicked, but the flow of vibrational energy through the protein and its coupling to the catalyzed bond activation need to be addressed. It is likely that only high-level theoretical studies like those conducted with WT and G121V *DHFR*s (3, 20–25, 30, 32, 71, 73) can fully interpret the role of tunneling and overall protein dynamics in catalysis at the molecular level.

The correlation between the genomic coupling (28) and the functional coupling arises despite the hydride transfer step for the WT *DHFR* is not rate-limiting under physiological conditions. However, mutations of conserved residues would turn that step into the rate-limiting step, as has been found in several mutants of *DHFR* (6, 33, 74) and offer an explanation as to how a hidden kinetic step may still impose evolutionary constraints.

Materials and Methods

All materials were purchased from Sigma (St. Louis, MO) unless otherwise indicated. 7,8-Dihydrofolate (H_2F) was prepared by dithionite reduction of folic acid as described by Blakely (75). All of the mixed-labeled cofactors ($R[4\text{-}^2H]\text{-NADPH}$, $R[4\text{-}^3H]\text{-NADPH}$, $[Ad\text{-}^{14}C]\text{-NADPH}$, $R[4,4\text{-}^2H,^3H]\text{-NADPH}$, and $[Ad\text{-}^{14}C, 4\text{-}^2H_2]\text{-NADPH}$) were synthesized as described (36, 37, 39, 40, 76).

WT *ecDHFR* and its mutants G121V, M42W, and G121V–M42W were expressed, purified, and stored as described (5, 33, 77).

The kinetic experiments and data processing procedures have been described in great detail (40, 41). In short, to measure the H/T KIE, NADPHs labeled with H or T at the 4R position were mixed and reacted with H_2F in the presence of the mutated DHFR under the conditions specified for each experiment. NADPH that was labeled with H was also labeled by ^{14}C in its adenosine ring to serve as a tracer for the conversion of these molecules. The reaction was quenched at different time points and at completion, and the depletion of the T in the product was analyzed as a function of fractional conversion to yield the KIE on the second-order rate constant k_{cat}/K_M . D/T KIEs were measured by using the same procedure but with D, instead of H, labeled NADPH. The observed H/T and D/T KIEs were used to calculate the intrinsic KIEs k_i/k_h , where k_i is the rate of the

C–H–C transfer with isotope i, and l or h represent the light or heavy isotope, respectively).

The isotope effects on the activation parameters for the intrinsic KIEs were calculated by nonlinear fit to the Arrhenius equation for KIEs:

$$k_l/k_h = A_l/A_h \cdot e^{\Delta E_{ah-l}/RT}. \quad [5]$$

Initial velocity measurements were performed at saturating substrate and cofactor concentrations over a temperature range of 5–40°C (40, 74). All experiments were conducted at high pH (adjusted at each experimental temperature) to ensure that the H transfer was mostly rate-limiting (2, 4, 33). The calculated steady-state rates (k_{cat}) for each temperature were fit to the Arrhenius equation through a nonlinear least-squares regression to obtain the activation parameters.

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